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<p>(21) International Application Number: PCT/US95/07178 (22) International Filing Date: 6 June 1995 (06.06.95) (30) Priority Data: 08/254,358 6 June 1994 (06.06.94) US (71) Applicant: CHILDREN'S HOSPITAL, INC. [US/US]; 700 Children's Drive, Columbus, OH 43205 (US). (72) Inventor: JOHNSON, Philip, R.; 1351 Bread Street, Gahanna, OH 43230 (US). (74) Agent: NOLAND, Greta, E.; Marshall, O'Toole, Gerstein, Murray &amp; Borun, 6300 Sears Tower, 233 South Wacker Drive, Chicago, IL 60606 (US).</p>		<p>(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i></p>
<p>(54) Title: RECOMBINANT AAV GENOME ENCODING IMMUNODEFICIENCY VIRUS PROTEIN (57) Abstract  The present invention provides adeno-associated virus (AAV) materials and methods which are useful for DNA delivery to cells. More particularly, the invention provides recombinant AAV (rAAV) genomes, comprising adeno-associated virus inverted terminal repeats flanking DNA sequences encoding an immunodeficiency virus protein operably linked to promoter and polyadenylation sequences, methods for packaging rAAV genomes, stable host cell lines producing rAAV and methods for delivering genes of interest to cells utilizing the rAAV. Particularly disclosed are rAAV useful in generating immunity to human immunodeficiency virus-1 and in therapeutic gene delivery for treatment of neurological disorders.</p>		

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Adeno-associated virus (AAV) is a replication-deficient parvovirus, the single-stranded DNA genome of which is about 4.7 kb in length including 145 nucleotide inverted terminal repeat (ITRs). See Figure 1. The nucleotide sequence of the AAV2 genome is presented in Srivastava *et al.*, *J. Virol.*, 45: 555-564 (1983).  
15 *Cis*-acting sequences directing viral DNA replication (*ori*), encapsidation/packaging (*pkg*) and host cell chromosome integration (*int*) are contained within the ITRs. Three AAV promoters, *p5*, *p19*, and *p40* (named for their relative map locations), drive the expression of the two AAV internal open reading frames encoding *rep* and  
20 *cap* genes. The two *rep* promoters (*p5* and *p19*), coupled with the differential splicing of the single AAV intron (at nucleotides 2107 and 2227), result in the production of four *rep* proteins (*rep* 78, *rep* 68, *rep* 52, and *rep* 40) from the *rep* gene. *Rep* proteins possess multiple enzymatic properties which are ultimately responsible for replicating the viral genome. The *cap* gene is expressed from the *p40*  
25 promoter and it encodes the three capsid proteins VP1, VP2, and VP3. Alternative and non-consensus translational start sites are responsible for the production of the

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three related capsid proteins. A single consensus polyadenylation site is located at map position 95 of the AAV genome. The life cycle and genetics of AAV are reviewed in Muzyczka, *Current Topics in Microbiology and Immunology*, 158: 97-129 (1992).

5                   When AAV infects a human cell, the viral genome integrates into chromosome 19 resulting in latent infection of the cell. Production of infectious virus does not occur unless the cell is infected with a helper virus (for example, adenovirus or herpesvirus). In the case of adenovirus, genes E1A, E1B, E2A, E4 and VA provide helper functions. Upon infection with a helper virus, the AAV provirus is  
10                   rescued and amplified, and both AAV and adenovirus are produced.

                  AAV possesses unique features that make it attractive as a vector for delivering foreign DNA to cells. AAV infection of cells in culture is noncytopathic, and natural infection of humans and other animals is silent and asymptomatic. Moreover, AAV infects most (if not all) mammalian cells allowing the possibility of  
15                   targeting many different tissues *in vivo*. Kotin *et al.*, *EMBO J.*, 11(13): 5071-5078 (1992) reports that the DNA genome of AAV undergoes targeted integration on chromosome 19 upon infection. Replication of the viral DNA is not required for integration, and thus helper virus is not required for this process. The AAV proviral genome is infectious as cloned DNA in plasmids which makes construction of  
20                   recombinant genomes feasible. Furthermore, because the signals directing AAV replication, genome encapsidation and integration are contained within the ITRs of the AAV genome, the internal approximately 4.3 kb of the genome (encoding replication and structural capsid proteins, rep-cap) may thus be replaced with foreign DNA such as a gene cassette containing a promoter, a DNA of interest and a  
25                   polyadenylation signal. Another significant feature of AAV is that it is an extremely

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stable and hearty virus. It easily withstands the conditions used to inactivate adenovirus (56° to 65° C for several hours), making cold preservation of rAAV-based vaccines less critical. Finally, AAV-infected cells are not resistant to superinfection.

Various groups have studied the potential use of AAV in treatment of disease states. Patent Cooperation Treaty (PCT) International Publication No. WO 91/18088 published November 28, 1991 and the corresponding journal article by Chatterjee *et al.*, *Science*, 258: 1485-1488 (1992) describe the transduction of intracellular resistance to human immunodeficiency virus-1 (HIV-1) in human hematopoietic and non-hematopoietic cell lines using an rAAV encoding an antisense RNA specific for the HIV-1 TAR sequence and polyadenylation signal. The review article Yu *et al.*, *Gene Therapy*, 1: 13-26 (1994) concerning gene therapy for HIV-1 infection lists AAV as a possible gene therapy vector for hematopoietic stem cells. The use of rAAV vectors as a delivery system for stable integration and expression of genes (in particular the cystic fibrosis transmembrane regulator gene) in cultured airway epithelial cells is described in PCT International Publication No. WO 93/24641 published December 9, 1993 and in the corresponding journal article by Flotte *et al.*, *Am. J. Respir. Cell Mol. Biol.*, 7: 349-356 (1992). Gene therapy involving rAAV in the treatment of hemoglobinopathies and other hematopoietic diseases and in conferring cell-specific multidrug resistance is proposed in PCT International Publication No. WO 93/09239 published May 13, 1993; Muro-Cacho *et al.*, *J. Immunol.*, 11: 231-237 (1992); LaFace *et al.*, *Viol.*, 162: 483-486 (1988); and Dixit *et al.*, *Gene*, 104: 253-257 (1991). Therapeutic gene delivery into glioma cells is proposed in Tenenbaum *et al.*, *Gene Therapy*, 1 (Supplement 1): S80 (1994).

A relatively new concept in the field of gene transfer is that immunization may be effected by the product of a transferee gene. Several attempts at "genetic immunization" have been reported including direct DNA injection of influenza A nucleoprotein sequences [Ulmer *et al.*, *Science*, 259: 1475-1749 (1993)],

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biolistic gun immunization with human growth hormone sequences [Tang *et al.*, *Nature*, 356: 152-154 (1992) and infection with retroviral vectors containing HIV-1 gp160 envelope protein sequences [Warner *et al.*, *AIDS RESEARCH AND HUMAN RETROVIRUSES*, 7(8): 645-655 (1991)]. While these approaches appear to be  
5 feasible, direct DNA inoculation may not provide long-lasting immune responses and serious questions of safety surround the use of retroviral vectors. The use of AAV for genetic immunization is a novel approach that is not subject to these problems.

An obstacle to the use of AAV for delivery of DNA is the lack of highly efficient schemes for encapsidation of recombinant genomes. Several methods  
10 have been described for encapsidating rAAV genomes to generate recombinant viral particles. These methods all require *in trans* AAV rep-cap and adenovirus helper functions. The simplest involves transfecting the rAAV genome into host cells followed by co-infection with wild-type AAV and adenovirus. See, for example, U.S. Patent No. 4,797,368 issued January 10, 1989 to Carter and Tratschin, and the  
15 corresponding journal article by Tratschin *et al.*, *Mol. Cell. Biol.*, 5(11): 3251-3260 (1985). This method, however, leads to unacceptably high levels of wild-type AAV. Another general strategy involves supplying the AAV functions on a second plasmid (separate from the rAAV genome) that is co-transfected with the rAAV plasmid. See, for example, Hermonat *et al.*, *Proc. Natl. Acad. Sci. USA*, 81: 6466-6470 (1984) and  
20 Lebkowski *et al.*, *Mol. Cell. Biol.*, 8(10): 3988-3996 (1988). If no sequence overlap exists between the two plasmids, then wild-type AAV production is avoided as is described in Samulski *et al.*, *J. Virol.*, 63(9): 3822-3828 (1989). This strategy is inherently inefficient, however, due to the requirement for three separate DNA transfer events (co-transfection of two plasmids as well as infection with adenovirus)  
25 to generate rAAV particles. Large scale production of rAAV by this method is costly and is subject to variations in transfection efficiency.

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Vincent *et al.*, *Vaccines*, 90: 353-359 (1990) reports that a cell line expressing rep-cap functions could be used to package rAAV. Such methods still requires transfection of the rAAV genome into the cell line and the resulting titer of rAAV reported was very low (only about  $10^3$  infectious units/ml). Dutton, *Genetic*  
5 *Engineering News*, 14(1): 1 and 14-15 (January 15, 1994) reports that Dr. Jane Lebkowski of Applied Immune Sciences manufactures rAAV using chimeric AAV/Epstein-Barr virus plasmids that contain a recombinant AAV genome, the hygromycin resistance gene and the EBV ori P fragment and EBNA gene. The plasmids are transfected into cells to generate stable cell lines. The stable cell lines  
10 are then transfected with wild-type AAV rep-cap functions and infected with adenovirus to produce rAAV. Like the method of Vincent, the Lebkowski packaging method requires both transfection and infection events to generate rAAV particles.

There thus exists a need in the art for efficient methods of packaging rAAV genomes and for specific rAAVs useful as vectors for DNA delivery to cells.

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#### SUMMARY OF THE INVENTION

The present invention provides recombinant AAV (rAAV) genomes useful for delivering non-AAV DNA of interest to a cell. The rAAV genomes of the invention include AAV ITRs flanking non-AAV DNA sequences of interest and lack rep-cap sequences encoding functional rep-cap proteins. If it is desirable to express  
20 the DNA of interest as a polypeptide in the cell, the rAAV genome also includes a (constitutive or regulatable) promoter and a polyadenylation signal operably linked to the DNA of interest to form a gene cassette. The gene cassette may also include intron sequences to facilitate processing of the RNA transcript in mammalian host cells. A presently preferred gene cassette includes the following DNA segments: (1)  
25 the cytomegalovirus (CMV) immediate early promoter, (2) the rabbit  $\beta$ -globin intron, (3) simian immunodeficiency virus (SIV) or human immunodeficiency (HIV) rev and

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envelope (gp160) genes, and (4) the rabbit  $\beta$ -globin polyadenylation signal. The rAAV genomes of the invention may be assembled in vectors useful for transfection of cells which are permissible for infection with a helper virus of AAV (e.g., adenovirus, E1-deleted adenovirus or herpesvirus). A vector of the invention which  
5 contains a rAAV genome including the foregoing preferred gene cassette, a neomycin resistance gene, and wild-type AAV rep-cap sequences was deposited in *E. coli* DH5 cells with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, on June 1, 1994 and was assigned ATCC Accession No. 69637.

10 Presently preferred rAAV genomes include the SIV rev and envelope (gp160) genes, or the HIV rev and envelope genes, as the non-AAV DNA(s) of interest. Also preferred are rAAV genomes which contain sequences encoding proteins which may ameliorate neurological disorders such as: sequences encoding  
15 nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), neurotrophins 3 and 4/5 (NT-3 and 4/5), glial cell derived neurotrophic factor (GDNF), transforming growth factors (TGF), and acidic and basic fibroblast growth factor (a and bFGF); sequences encoding tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase (AADC); sequences  
20 encoding superoxide dismutase (SOD 1 or 2), catalase and glutathione peroxidase; sequences encoding interferons, lymphokines, cytokines and antagonists thereof such as tumor necrosis factor (TNF), CD4 specific antibodies, and TNF or CD4 receptors; sequences encoding GABA receptor isoforms, the GABA synthesizing enzyme glutamic acid decarboxylase (GAD), calcium dependent potassium channels or ATP-sensitive potassium channels; and sequences encoding thymidine kinase. Also  
25 contemplated by the invention are rAAV genomes including globin, oncogene, ras, and p53 sequences. Recombinant AAV genomes including antisense nucleotides that



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affect expression of certain genes such as cell death suppressor genes (*e.g.*, bcl-2) or that affect expression of excitatory amino acid receptors (*e.g.*, glutamate and NMDA receptors) are also contemplated for modulating neurological disorders.

Other DNA sequences of interest contemplated by the invention include  
5 sequences from pathogens including: HIV-1 and HIV-2 (sequences other than rev and gp160 sequences); human T-lymphotrophic virus types I and II; respiratory syncytial virus; parainfluenza virus types 1-4; measles virus; mumps virus; rubella virus; polio viruses types 1-3; influenza virus types A, B and C; non-human influenza viruses (avian, equine, porcine); hepatitis virus types A, B, C, D and E; rotavirus; norwalk  
10 virus; cytomegaloviruses; Epstein-Barr virus; herpes simplex virus types 1 and 2; varicella-zoster virus; human herpes virus type 6; hantavirus; adenoviruses; chlamydia pneumoniae; chlamydia trachomatis; mycoplasma pneumoniae; mycobacterium tuberculosis; atypical mycobacteria; feline leukemia virus; feline immunodeficiency virus; bovine immunodeficiency virus; equine infectious anemia virus; caprine  
15 arthritis encephalitis virus; and visna virus.

Cell lines of the invention are stably transfected with both rAAV genomes of the invention and with copies of the AAV rep and cap genes. Preferred cell lines are mammalian cell lines, for example, HeLa cell lines. Infection of the cell lines of the invention with AAV helper virus results in packaging of the rAAV  
20 genomes as infectious rAAV particles. A presently preferred stable cell line is the A64 HeLa cell line which was deposited with the ATCC on June 1, 1994 and was assigned ATCC Accession No. CRL 11639. The present invention also provides stable cell lines containing AAV rep and cap sequences but no rAAV genome.

Recombinant AAV generated by the foregoing packaging process are  
25 useful for delivering the DNA of interest to cells. *In vivo*, rAAV may be used as antisense delivery vectors, gene therapy vectors or vaccine (*i.e.*, genetic immunization) vectors. Treatment of disease conditions including, for example,

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AIDS; neurological disorders including cancer, Alzheimer's disease, Parkinson's disease, Huntington's disease, and autoimmune diseases such as multiple sclerosis, trauma, depression, migraine, pain or seizure disorders; adult T-cell leukemia; tropical spastic paraparesis; upper and lower respiratory tract infections; upper and lower respiratory tract infections; measles; mumps; rubella; polio; influenza; influenza; hepatitis; hepatitis; hepatitis; hepatitis; hepatitis; diarrhea; diarrhea; systemic cytomegalovirus infections; mononucleosis-like illness; systemic Epstein-Barr virus infections; classic infectious mononucleosis; systemic herpes simplex types 1 and 2 infections; genital herpes simplex infections; chickenpox; roseola; febrile illness due to human herpes virus type 6; pneumonia and adult respiratory distress syndrome; upper and lower respiratory tract infections; conjunctivitis; upper and lower respiratory tract infections; upper and lower respiratory tract infections; genital tract infections; upper and lower respiratory tract infections; pulmonary and extrapulmonary tuberculosis; systemic infections due to atypical mycobacteria; feline leukemia; feline AIDS; bovine AIDS; equine infectious anemia; arthritis and encephalitis in goats; and pneumonia and encephalitis in sheep are contemplated by the invention. As a vaccine vector, rAAV delivers a gene of interest to a cell and the gene is expressed in the cell. The vaccine vectors may be used to generate intracellular immunity if the gene product is cytoplasmic (e.g., if the gene product prevents integration or replication of a virus). Alternatively, extracellular/systemic immunity may be generated if the gene product is expressed on the surface of the cell or is secreted.

A host (especially a human host) may be immunized against a polypeptide of a disease-causing organism by administering to the host an immunity-inducing amount of a rAAV of the invention which encodes the polypeptide. Immunization of a human host with a rAAV of the invention involves administration by inoculation of an immunity-inducing dose of the virus by the parenteral route

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(*e.g.*, by intravenous, intramuscular or subcutaneous injection), by surface scarification or by inoculation into a body cavity. Typically, one or several inoculations of between about 1000 and about 10,000,000 infectious units each, as measured in susceptible human or nonhuman primate cell lines, are sufficient to effect immunization of a human host. Virus to be used as a vaccine may be utilized in liquid or freeze-dried form (in combination with one or more suitable preservatives and/or protective agents to protect the virus during the freeze-drying process). For gene therapy (*e.g.*, of neurological disorders which may be ameliorated by a specific gene product) a therapeutically effective dose of a rAAV of the invention which encodes the polypeptide is administered to a host in need of such treatment. The use of rAAV of the invention in the manufacture of a medicament for inducing immunity in, or providing gene therapy to, a host is contemplated.

#### **BRIEF DESCRIPTION OF THE DRAWING**

Numerous other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description thereof, reference being made to the drawing wherein:

Figure 1 is a schematic representation of the AAV genome;

Figure 2 is a schematic representation of plasmid psub201 which was the source of AAV2 sequences utilized in the examples;

Figure 3A through 3B is a flow diagram of the construction of a rAAV genome of the invention in vector pAAV/DMV/SIVrev-gp160;

Figure 4 is a flow diagram of the construction of the vector pAAV/CMV/SIVrev-gp160/neo/rep-cap useful to generate a stable cell line producing rAAV of the invention; and

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Figure 5 is a schematic representation of a method for packaging rAAV utilizing stable host cell lines of the invention.

### DETAILED DESCRIPTION OF THE DRAWING

The present invention is illustrated by the following examples relating to the production and use of rAAV of the invention. Example 1 describes the construction of a vector including a rAAV genome containing the SIV rev and envelope (gp160) genes, while Example 2 describes the construction of a vector including the AAV rep-cap genes and a neomycin resistance gene. Example 3 sets out the construction of a vector to be used to generate stable cell lines producing rAAV from the vectors described in Examples 1 and 2. The generation of stable cell lines producing rAAV encoding the SIV rev and gp160 proteins is detailed in Example 4. Example 5 sets out a preferred procedure for purifying rAAV from stable cell lines of the invention. Example 6 describes the generation of stable cell lines expressing the AAV rep-cap genes. Example 7 presents results of infection of various mammalian cells and cell lines with the rAAV described in Example 4 which show that gp160 protein is expressed in the infected cells. Example 8 describes the generation of stable cell lines producing a rAAV that includes the  $\beta$ -galactosidase gene as a DNA of interest and that is useful as a positive control virus for expression of a DNA of interest in target cells or tissues. Example 9 presents the results of experiments in which rAAV of the invention was used to express a DNA of interest *in vivo*. Example 10 describes methods contemplated by the invention for increasing the titer of rAAV produced by stable cell lines.

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Example 1

A vector including a rAAV genome containing a SIV rev and envelope (gp160) gene cassette was constructed from an existing plasmid designated psub201 [Samulski *et al.*, *supra*]. Figure 2 is a diagram of plasmid psub201 wherein restriction endonuclease sites are shown and abbreviated as follows: P, PvuII; X, XbaI; B, BamHI; H, HindIII; and N, NaeI. The plasmid contains a modified wild-type AAV2 genome cloned between the PvuII restriction sites. The DNA sequence of the wild-type AAV2 genome is set out in SEQ ID NO: 1. The AAV2 sequence was modified to include convenient restriction sites. Specifically, two XbaI restriction sites were added via linker addition at sequence positions 190 and 4484. These sites are internal to 191 bp inverted terminal repeats (ITRs) which included the 145 bp ITRs of the AAV genome. The insertion of these sites allows the complete removal of the internal 4.3 kb fragment containing the AAV rep-cap genes upon XbaI digestion of the plasmid. In Figure 2, the 191 bp ITRs are designated by inverted arrows.

The rAAV genome vector of the invention (pAAV/CMV/SIVrev-gp160) was generated in several steps.

First, plasmid psub201 was digested with XbaI and the approximately 4 kb vector fragment including the AAV ITRs was isolated. A CMV gene expression cassette was then inserted between the AAV ITRs by blunt end ligation. The CMV expression cassette was derived as a 1.8 kb XbaI - AflIII DNA fragment from the vector pCMV-NEO-BAM described in Karasuyama *et al.*, *J. Exp. Med.*, 169: 13-25 (1989). Prior to ligation, the molecular ends were filled in using the Klenow fragment of DNA polymerase I. The CMV expression cassette contained a 750 bp portion of the CMV immediate early promoter, followed by a 640 bp intron and a 360 bp polyadenylation signal sequence which were derived from the rabbit  $\beta$ -globin gene. Between the intron and poly A sequences were two cloning sites: a unique

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BamHI site and two flanking EcoRI restriction sites. The resulting vector was named pAAV/CMV. See Figure 3A wherein restriction endonuclease cleavage sites are shown and abbreviated as follows: B, BamHI; E, EcoRI; N, NaeI; and P, PvuII.

Second, the pAAV/CMV expression vector was linerized at the BamHI  
5 site and sticky ends were blunted with Klenow. A PCR-generated, 2.7 kb SIV subgenomic fragment containing the rev and envelope (gp160) sequences [SEQ ID NO: 2, Hirsch *et al.*, *Nature*, 339: 389-392 (1989)] was cloned into the blunt-ended BamHI site. The resulting recombinant AAV genome vector, pAAV/CMV/SIVrev-gp160, is 8.53 kb in length. See Figure 3B wherein restriction endonuclease cleavage  
10 sites are shown and abbreviated as follows: N, NaeI and P, PvuII. The vector contains the following DNA segments in sequence: (1) an AAV ITR, (2) the CMV promoter, (3) the rabbit  $\beta$ -globin intron, (4) the SIV rev and envelope sequences, (5) the rabbit  $\beta$ -globin polyadenylation signal, and (6) an AAV ITR. In transient transfection assays of human 293 cells, this vector resulted in high levels of  
15 expression of the SIV gp160 protein as determined by radioimmunoprecipitation assays using polyclonal sera from monkeys infected with SIV.

The invention specifically contemplates substitution by standard recombinant DNA techniques of the following sequences for the SIV rev/envelope sequences in the foregoing vector: HIV-1 rev/envelope sequences (the HIV-1<sub>MN</sub> rev/envelope sequence is set out in SEQ ID NO: 3); nerve growth factor [Levi-Montalcini, *Science*, 237: 1154-1162 (1987)]; ciliary neurotrophic factor [Manthorpe  
20 *et al.*, beginning at p. 135 in *Nerve Growth Factors*, Wiley and Sons (1989)]; glial cell derived neurotrophic factor [Lin *et al.*, *Science*, 260: 1130-1132 (1993)]; transforming growth factors [Puolakkainen *et al.*, beginning at p. 359 in *Neurotrophic Factors*, Academic Press (1993)]; acidic and basic fibroblast growth factors [Unsicker  
25 *et al.*, beginning at p. 313 in *Neurotrophic Factors*, Academic Press (1993)]; neurotrophin 3 [Maisonpierre *et al.*, *Genomics*, 10: 558-568 (1991)]; brain-derived

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neurotrophic factor [Maisonpierre, *supra*]; neurotrophin 4/5 [Berkemeier *et al.*, *Neuron*, 7: 857-866 (1991)]; tyrosine hydroxylase [Grima *et al.*, *Nature*, 326: 707-711 (1987)]; and aromatic amino acid decarboxylase [Sumi *et al.*, *J. Neurochemistry*, 55: 1075-1078 (1990)].

5

### Example 2

A plasmid designated pSV40/neo/rep-cap which contains the AAV rep-cap genes and a neomycin resistance gene was constructed to be used in conjunction with the rAAV genome vector described in Example 1 to generate a stable cell line producing rAAV.

10

A plasmid designated pAAV/SVneo (Samulski *et al.*, *supra*) was digested with EcoRI and BamHI to release a 2.7 kb insert including a 421 bp portion of the SV40 early promoter, a 1.4 kb neomycin resistance gene, and a 852 bp DNA fragment containing the SV40 small t splice site and SV40 polyadenylation signal. This released insert was cloned into the EcoRI and BamHI sites of pBLUESCRIPT KS+ (Stratagene, La Jolla, CA) to generate the 5.66 kb plasmid pSV40/neo. Next, the approximately 4.3 kb DNA fragment containing the AAV rep-cap genes, derived from the digestion of psub201 with XbaI as described in Example 1, was ligated into the XbaI restriction site of pSV40/neo to create the plasmid pSV40/neo/rep-cap (about 10 kb). The construction of this plasmid is detailed in first half of Figure 4 wherein restriction endonuclease sites are shown and abbreviated as follows: B, BamHI; E, EcoRI; HindIII; P, PvuII; N, NotI; RV, EcoRV; and X, XbaI. This plasmid was functional in transient assays for rep and cap activity and was itself ultimately used to derive stable cell lines (see Example 5 below).

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### Example 3

A final vector to be used to generate stable cell lines producing rAAV was generated from vector pAAV/CMV/SIVrev-gp160 (Example 1) and plasmid pSV40/neo/rep-cap (Example 2).

5           The construction entailed removing the neo-rep-cap gene cassette from pSV40/neo/rep-cap and inserting it into a unique NaeI site in pAAV/CMV/SIVrev-gp160 (see Figure 3B). Specifically, vector pAAV/CMV/SIVrev-gp160/neo/rep-cap was made by agarose gel band isolating a 7.0 kb EcoRV-NotI DNA fragment containing the SV/neo and rep-cap expression domains from pSV40/neo/rep-cap. The  
10       sticky ends of the fragment were blunted with Klenow and the fragment was ligated into the blunt-ended NaeI site of pAAV/CMV/SIVrev-gp160. See Figure 4. Vector pAAV/CMV/SIVrev-gp160/neo/rep-cap (ATCC 69637) contains the following elements: (1) the rAAV genome; (2) AAV rep-cap genes; and (3) the neomycin resistance gene.

15

### Example 4

The vector pAAV/CMV/SIVrev-gp160/neo/rep-cap was used to generate stable cells lines containing both the rAAV genome of the invention and AAV rep-cap genes.

HeLa cells at 70% confluency were transfected with 10  $\mu$ g of  
20       pAAV/CMV/SIVrev-gp160/neo/rep-cap plasmid DNA in 100 mm dishes. Cells were transfected for 6 hours after formation of DOTAP/DNA complexes in serum minus media as prescribed by the manufacturer's protocol (Boehringer-Mannheim, Indianapolis, IN). Following the removal of the transfection medium, DMEM media containing 10% fetal bovine serum was added to the cells. Three days later, media  
25       supplemented with 700  $\mu$ g/ml Geneticin (Gibco-BRL, Gaithersburg, MD) was used to select for cells that stably expressed the neomycin resistance gene. Fresh Geneticin



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containing DMEM media was added every four days. Geneticin resistant clones were selected 10-14 days after selective media was added. A total of fifty-five colonies were selected and transferred to 24-well plates and expanded for further analysis.

5 The fifty-five neomycin resistant HeLa cell lines were initially screened for functional rep gene activity; twenty-one scored positive. Rep gene activity was assayed by infecting the cell lines with adenovirus type 5 (Ad5). Infection by adenovirus transactivates the rep and cap genes. This results in the replication of the rAAV genome and subsequent encapsidation of these sequences into infectious rAAV particles. A schematic representation of rAAV production is shown in Figure 5.  
10 Following maximum Ad5-induced cytopathic effect (CPE; rounding of cells and 90% detachment from the culture flask), cell lysates were prepared and Hirt DNA (low molecular weight DNA) was isolated [Hirt, *J. Mol. Biol.*, 26: 365-369 (1967)]. Southern blot analysis was used to visualize the synthesis of recombinant AAV (rAAV) replicative forms (single strand, monomeric, and dimeric forms). Control  
15 wells not receiving Ad5 were always negative. Cell lines with high relative levels of rep gene activity were selected for further study.

To assay for cap gene functionality, cell lines were infected with Ad5 and clarified lysates prepared after the development of maximum CPE. The cell lysates, Ad5, and wild-type AAV were used to infect HeLa cells. Following the  
20 development of Ad5 induced CPE (72 hr), Hirt DNA was isolated and Southern blot analysis performed. Cell line lysates that gave rise to gp160 hybridizable rAAV (SIV gp160) replicative sequences were scored positive for capsid production.

An infectious unit/ml (IU/ml) titer of rAAV produced by each cell line was derived by co-infecting C12 cells (exhibiting stable rep and cap gene expression)  
25 with Ad5 and a serial ten-fold dilution of the clarified cell line lysate to be tested. After maximum Ad5-induced CPE, Hirt DNA was isolated and Southern blot analysis

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performed to detect the presence of rAAV replicative forms. The end-point dilution that produced visible monomeric and dimeric replication intermediates was taken as the titer. Titer estimation was based on two to four replicate experiments.

Results of characterization of eight of the fifty-five cell lines are shown in Table 1 below wherein "ND" indicates a value was not determined.

TABLE 1

Cell Line	Rep Function	Cap Function	Titer (IU/ml)
A5	++	+	$10^4$
A11	++++	+	$10^5$
A15	++++	+	$10^5$
A37	++++	+	ND
A60	+++++	-	$<10^1$
A64	+++++	+	$10^6$
A69	++	-	ND
A80	++++	+	$10^5$

Cell line A64 (ATCC CRL 11639) produced a high titer of rAAV ( $10^6$  iu/ml) in clarified lysates. This titer is approximately 1000-fold higher than the titer of rAAV reported by Vincent *et al.*, *supra*.

The rAAV produced by the various cell lines was also tested for its ability to express SIV gp160 in HeLa cells infected with the recombinant virus. Concentrated stocks of rAAV produced by the eight stable cell lines listed in Table 1 were generated. Cell lysates containing rAAV particles were subjected to step

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density gradient (CsCl) purification. After desalting dialysis and heat-inactivation of Ad5, the rAAV particles were used to infect (transduce) HeLa cells in culture. Two lines of investigation were pursued. First, the transduced cells were tested for the presence of SIV gp160-specific mRNA by performing RT-PCR on total RNA  
5 collected 72 hours after transduction. Primers specific for SIV gp160 amplified a predicted 300 bp fragment only in the presence of reverse transcriptase and Taq polymerase; samples run without reverse transcriptase were uniformly negative. Second, HeLa cells were transduced with various dilutions of the same rAAV/SIV stock as described above and, at 72 hours post transduction, indirect  
10 immunofluorescence was performed on the infected cells. At all dilutions tested (out to 1:200), cells positive for the SIV gp160 protein were detected; lower dilutions clearly had more positive cells.

The A64 cell line was tested for wild-type AAV production by a standard method. The cell line was infected with adenovirus to produce rAAV as a  
15 lysate. The lysate was then used to infect normal HeLa cells either: (i) alone; (ii) with adenovirus; or (iii) with adenovirus and wild-type AAV. As a control, HeLa cells were infected with adenovirus and wild-type AAV without rAAV. Hirt DNA was prepared and analyzed by Southern blotting (two different blots) for replicating forms of either rAAV or wild-type AAV. No wild-type AAV was detected in A64  
20 cells not exposed to wild-type AAV.

Because the present invention involves the establishment of stable cell lines containing not only copies of the AAV rep and cap genes, but also of the rAAV genome (with ITRs flanking DNA of interest), rAAV is produced by merely infecting the cell line with adenovirus. Transfection of exogenous DNA is not required,  
25 thereby increasing the efficiency of rAAV production compared to previously

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described methods. Other significant features of the invention are that no wild-type AAV is produced and that scale-up for production of rAAV is easy and is limited only by normal constraints of cell growth in culture.

#### Example 5

5           A method to isolate and purify rAAV from stable (producer) cell lines was developed.

          Producer cells (for example, the A64 cells of Example 4) were seeded at a cell density of  $3 \times 10^6$  producer cells per  $175 \text{ cm}^2$  surface area in growth medium. Cells reached a density of about  $8 \times 10^6$  cells after 16-18 hours, and were  
10 then infected with adenovirus (Ad5) at a multiplicity of infection (moi) of 5 for 1-2 hours in growth medium. A 15 ml infection volume was used, and after the 1-2 hour infection, 10 ml of growth medium was added to each flask to obtain a final volume of 25 ml. [Alternatively, Ad5 may be added directly by: removing all but 15 ml of growth medium and adding Ad5 in a volume of 10 ml (diluted in HBSS) to give a  
15 final volume of 25 ml.]

          Cells were harvested at about 48-60 hours after infection when most cells released from the flask after a vigorous shake. The cells were then stained with trypan blue to determine the percentage of viable cells. It is desirable for greater than 80% to be viable. Cells were then transferred to 250 ml disposable conical bottles  
20 (Corning) and pelleted at  $1000 \times g$  for 15 minutes at  $4^\circ\text{C}$ . The resulting supernatant was removed saving an aliquot and the cells were suspended in TM buffer (50mM Tris, pH 8.0, and 1mM  $\text{MgCl}_2$ ) at a density of  $5 \times 10^6$  cells/ml. The cells were subjected to three cycles of freeze/thaw on dry ice with vortexing 2 minutes between each thaw. The lysed cells were then heated to  $56^\circ\text{C}$  for 30 minutes to 1 hour with  
25 vortexing every 7.5 minutes during the last thaw. Ten percent deoxycholate was added to the lysate to a final concentration of 1%, and the mixture was incubated at

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37°C for 30 minutes with intermittent vortexing to achieve complete lysis. If necessary to achieve complete lysis, the mixture was sonicated 3 times on maximum setting for 2 minutes each time. A hemocytometer was used to confirm complete cell lysis. Cell debris was pelleted at 2000 x g for 15 minutes at 4°C. The rAAV  
5 containing supernatant was saved.

The rAAV was isolated using a 1.31 g/ml CsCl cushion. Twenty-one ml of lysate supernatant was layered on a 14 ml CsCl cushion in a SW-28 tube, and spun 16,000 rmp, 10°C for 16 hours. The resulting supernatant was aspirated and the rAAV pellet was washed with HBSS to remove residual CsCl. The pAAV pellet  
10 was re-dissolved in 20mM Tris pH 8, 150mM NaCl, 1mM MgCl<sub>2</sub> (TMN buffer) in the smallest volume manageable (about 500 µl/pellet) and let hydrate overnight. It was then heated to 56°C for 30 minutes with vortexing every 5 minutes. At this endpoint, the virus was dialyzed against the TMN buffer to remove all traces of cesium if the virus was not going to be further purified.

15 The rAAV may be further purified by isopycnic banding. This is appropriate under conditions in which the virus is to be administered *in vivo*. The hydrated rAAV was brought up to a CsCl density of 1.41 g/ml, and then spun in an SW-41 tube at 30K for 48 hours at 10°C. The top portion of the gradient containing adenovirus (density 1.34 - 1.36 g/ml) was discarded and the remaining portion of the  
20 CsCl gradient was diluted with TMN down to a buoyant density of less than 1.1 g/ml. rAAV was then pelleted by an overnight spin at > 60,000 x g. The rAAV was resuspended in a minimal amount of TMN buffer supplemented with 1% gelatin. For efficient hydration, the pellet was allowed to sit overnight at 4°. The rAAV was then aliquoted and stored at 20°C.

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Example 6

Concurrent with the generation of the stable cells described in Example 4, stable HeLa cell lines were established by similar methods which contained rep-cap genes but no rAAV genome using plasmid pSV40/neo/rep-cap (Example 2). A total  
5 of fifty-two neomycin resistant HeLa cell lines were isolated and characterized.

To test for rep gene function, each cell line was infected with Ad5 and subsequently transfected with pAAV/CMV/SIVrev-gp160. Following Ad5-induced CPE (72 hr), Hirt DNA was isolated and Southern blot analysis performed. Rep gene function was scored positive for cell lines that produced monomeric and dimeric  
10 rAAV gp160 sequences. The intensity of autoradiographic signal was used as a relative measure of rep gene expression (1-5+). Ad5 minus control samples never produced rAAV replicative forms. Cap gene proficiency was assayed in a similar manner (Ad5 infection and pAAV/CMV/SIVrev-gp160 transfection), except that a clarified cell lysate was prepared after the development of maximum CPE. HeLa  
15 cells were then co-infected with a portion of the clarified cell lysate, Ad5, and wild-type AAV. Hirt DNA was isolated 72 hours later, and hybridization analysis was used to visualize the existence of rAAV/gp160 replicative forms (monomeric and dimeric). In the assay described, the C12 cell line yielded the highest relative proportion of rAAV/gp160/120 sequences.

20 Results of the characterization assays are presented for eight cell lines are presented in Table 2 wherein the abbreviation "ND" indicates that a value was not determined.

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TABLE 2

	Cell Line	Rep Function	Cap Function
	C2	+++++	+
	C12	++++	+++
5	C16	-	ND
	C18	+++	ND
	C23	+++	ND
	C25	+++	-
	C27	++	ND
10	C44	++++	+

There are two principal uses for the stable cell lines expressing rep-cap sequences: (1) generating rAAV particles if the cell lines are transfected with a rAAV genome and infected with helper virus; and (2) determining rAAV infectious titers. To estimate rAAV infectious titers, these cell lines are co-infected with adenovirus and serial dilutions of the rAAV stock. After maximum CPE, Hirt DNA is isolated and replicative rAAV forms are visualized by Southern blot analysis. End point titration (last rAAV stock dilution to give positive hybridization signal) is then used to determine the infectious titer.

#### Example 7

The ability of the rAAV produced by HeLa cell line A64 to infect (transduce) and produce SIV gp160 protein in various mammalian cell types in addition to HeLa cells (see Example 4) was assayed. The rAAV (at a multiplicity of

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infection of approximately 1) was used to infect cells either in a monolayer or in suspension, depending on the cell type. Three days after rAAV infection, the cells were fixed in acetone/methanol and evaluated for the production of gp160 by indirect immunofluorescence using polyclonal antisera from an SIV-infected monkey. The following cells or cell lines were infected and shown to produce gp160; fetal rat brain cells (neurons and glial cells), mouse 3T3 fibroblasts, mouse vagina, human vagina, human colon, human and monkey lymphocytes and 293 cells. No non-permissive cell type was identified. These results demonstrate that the rAAV produced by the A64 cell line infects a wide range of mammalian cell types and leads to cell surface expression of the SIV envelop gene product, gp160, in the transduced cells.

#### Example 8

Stable cell lines were generated that produced rAAV carrying the  $\beta$ -galactosidase gene as a gene of interest. These rAAV are useful as positive control to test for expression of a DNA of interest in a target cell or tissue.

A vector like pAAV/CMV/SIVrev-gp160/neo/rep-cap was constructed that included a  $\beta$ -galactosidase gene expression cassette (Clontech, Palo Alto, CA) containing the human CMV promoter, the *E. coli*  $\beta$ -galactosidase gene, and the SV-40 splice/polyadenylation sequence instead of the rabbit  $\beta$ -globin intron, SIV rev and envelope sequences, and rabbit  $\beta$ -globin polyadenylation signal between the AAV ITRs. This  $\beta$ -galactosidase gene expression cassette was cloned in between the AAV ITRs by standard recombinant methods.

Stable HeLa cell lines which produced rAAV containing the  $\beta$ -galactosidase gene (rAAV/ $\beta$ -gal) were generated as described in Example 4 using the foregoing vector.



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### Example 9

The rAAV/ $\beta$ -gal of Example 8 were used to demonstrate the use of rAAV of the invention for gene transfer into the brains of live mice. rAAV/ $\beta$ -gal was injected directly into the brains of mice and the brains were then examined for  
5 evidence of  $\beta$ -galactosidase activity.

Balb/c mice ( $n = 3$ ; male; 9 months old) were anesthetized and secured on a murine stereotactic platform. Using sterile technique, rAAV/ $\beta$ -gal ( $1 \mu\text{l}$  containing  $3 \times 10^6$  infectious units) was injected into the right hippocampus. Additional mice ( $n = 3$ ) received an injection of diluent as controls. One week after  
10 injection, mice were sacrificed by cardiac exsanguination followed by sequential infusion of 50 ml of heparinized phosphate buffered saline, then 50 ml of a mixture of paraformaldehyde (0.5%) and glutaraldehyde (2.5%) in 0.1M phosphate buffer (pH 7.3). Whole brains were removed, post-fixed in the same fixative mixture (2 hours) and frozen in O.C.T. Cryostat sections ( $10 \mu\text{m}$ ) were placed on poly-L-lysine coated  
15 microscope slides and stored at  $-20^\circ\text{C}$ . Slides were thawed at room temperature, fixed again (5 minutes at  $4^\circ\text{C}$ ), washed twice in PBS, and transferred to X-gal stain (a substrate for the enzymatic activity of  $\beta$ -galactosidase). After incubation overnight at  $37^\circ$ , slides were washed twice in PBS, counterstained with nuclear fast red, and examined microscopically for blue-stained cells (cells where  $\beta$ -galactosidase was  
20 being expressed).

In the brains of the mice injected with rAAV/ $\beta$ -gal, blue-stained cells in the hippocampus were easily detected upon microscopic examination. In the brains of mice injected with diluent (controls), no blue-stained cells were found.

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Example 10

Various methods to increase the titer of rAAV generated from stable cell lines which involve providing additional AAV rep and cap genes to the cell lines are contemplated by the invention.

5 In a first method which demonstrates the usefulness of providing additional rep and cap genes, a producer cell line is transfected with a plasmid containing a helper plasmid carrying AAV rep and cap genes prior to adenovirus infection. Results from experiments in which a rAAV/ $\beta$ -gal producer cell line (H44) was so transfected are presented in Table 3 below.

10

TABLE 3

Treatment	Viral Yield	IU/cell	Fold increase
Mock transfection	$7 \times 10^7$	7	0
50 $\mu$ g pBS/rep-cap	$1 \times 10^9$	100	14
100 $\mu$ g pBS/rep-cap	$8 \times 10^8$	80	11
15 150 $\mu$ g pBS/rep-cap	$1 \times 10^9$	110	16

In a second method, the AAV rep and cap genes are placed on a separate plasmid containing an EBV or BPV origin of DNA replication and a drug resistance marker (hygromycin). The plasmid will be transfected into a producer cell line and new cell lines are then selected on neomycin and hygromycin. This selection  
20 pressure will result in stable cell lines which contain both rAAV genomes and multiple copies of the AAV rep and cap genes.

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In a third method, the AAV rep and cap genes are cloned into the adenovirus genome in the E3 location under the control of the tetracycline operator.

While the present invention has been described in terms of preferred embodiments, it understood that variations and improvements will occur to those  
5 skilled in the art. Therefore, only such limitations as appear in the claims should be placed on the invention.

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SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Johnson, Philip R.
- (ii) TITLE OF INVENTION: Adeno-Associated Virus Materials and Methods
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
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  - (F) ZIP: 60606
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
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  - (A) APPLICATION NUMBER: US 08/254,358
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  - (C) CLASSIFICATION:
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## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 4680 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTGGCCACTC CCTCTCTGCG CGCTCGCTCG CTCACTGAGG CCGGGCGACC AAAGGTCGCC	60
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CCAGCAATCG GATTTATAAA ATTTTGGAAC TAAACGGGTA CGATCCCCAA TATGCGGCTT	1260
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ACGGGTGCGT AAAGTGGACC AATGAGAACT TTCCCTTCAA CGACTGTGTC GACAAGATGG	1440
TGATCTGGTG GGAGGAGGGG AAGATGACCG CCAAGGTCGT GGAGTCGGCC AAAGCCATTC	1500

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TCGGAGGAAG CAAGGTGCGC GTGGACCAGA AATGCAAGTC CTCGGCCCAG ATAGACCCGA	1560
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CTTCCAGATT GGCTCGAGGA CACTCTCTCT GAAGGAATAA GACAGTGGTG GAAGCTCAAA	2280
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GCAGACGTCT	TCATGGTGCC	ACAGTATGGA	TACCTCACCC	TGAACAACGG	GAGTCAGGCA	3360
GTAGGACGCT	CTTCATTTTA	CTGCCTGGAG	TACTTTCCTT	CTCAGATGCT	GCGTACCGGA	3420
AACAACCTTA	CCTTCAGCTA	CACTTTTGAG	GACGTTCCCT	TCCACAGCAG	CTACGCTCAC	3480
AGCCAGAGTC	TGGACCGTCT	CATGAATCCT	CTCATCGACC	AGTACCTGTA	TTACTTGAGC	3540
AGAACAAACA	CTCCAAGTGG	AACCACCACG	CAGTCAAGGC	TTCAGTTTTTC	TCAGGCCGGA	3600
GCGAGTGACA	TTCGGGACCA	GTCTAGGAAC	TGGCTTCCTG	GACCCTGTTA	CCGCCAGCAG	3660
CGAGTATCAA	AGACATCTGC	GGATAACAAC	AACAGTGAAT	ACTCGTGGAC	TGGAGCTACC	3720
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CAGGACAGAG	ATGTGTACCT	TCAGGGGCCC	ATCTGGGCAA	AGATTCCACA	CACGGACGGA	4080
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GCTTCCTTCA	TCACACAGTA	CTCCACGGGA	CACGGTCAGC	GTGGAGATCG	AGTGGGAGCT	4260
GCAGAAGGAA	AACAGCAAAC	GCTGGAATCC	CGAAATTCAG	TACACTTCCA	ACTACAACAA	4320
GTCTGTAAAT	CGTGGACTTA	CCGTGGATAC	TAATGGCGTG	TATTCAGAGC	CTCGCCCCAT	4380
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CGTTGCAGTT	GAACCTTGGT	CTCTGCGTAT	TTCTTTCTTA	TCTAGTTTCC	ATGGCTACGT	4500
AGATAATTAG	CATGGCGGGT	TAATCATTA	CTACAAGGAA	CCCCTAGTGA	TGGAGTTGGC	4560
CACTCCCTCT	CTGCGCGCTC	GCTCGCTCAC	TGAGGCCGGG	CGACCAAAGG	TCGCCCCGACG	4620
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## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2658 base pairs

(B) TYPE: nucleic acid

- 30 -

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGGGATGTC TTGGGAATCA GCTGCTTATC GCGCTCTTGC TAGTAAGTGT TTTAGAGATT	60
TGTTGTGTTC AATATGTAAC AGTATTCTAT GGTGTACCAG CATGGAAGAA TGCGACAATT	120
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AATGATGATT ACTCAGAATT GGCAATCAAT GTCACAGAGG CTTTGTATGC TTGGGATAAT	240
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TGGGGTTTGA CAGGAAACGC AGGGACAACA ACAACAGCAA TAACAACAAC AGCAACACCA	420
AGTGTAGCAG AAAATGTTAT AAATGAAAGT AATCCGGGCA TAAAAATAA TAGTTGTGCA	480
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AATGAAAGTG AGAGTAAATG TTACATGCAT CATTGTAACA CCAGTGTTAT TCAAGAATCC	660
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GTTTCTTCAT GCACAAGAAT GATGGAGACG CAAACCTCTA CTTGGTTTGG CTTCAATGGT	840
ACTAGGGCAG AAAATAGAAC ATACATTTAT TGGCATGGCA AAAGTAATAG AACCATAATT	900
AGCTTGAATA AGTATTATAA TCTAACAATG AGATGTAGAA GACCAGAAAA TAAGACAGTT	960
TTACCAGTCA CCATTATGTC AGGGTTGGTC TTCCATTCGC AGCCCATAAA TGAGAGACCA	1020
AAACAGGCCT GGTGCTGGTT TGAAGGAAGC TGGAAGGAGG CCATCCAGGA AGTGAAGGAA	1080
ACCTTGGTCA AACATCCCAG GTATACGGGA ACTAATGATA CTAGGAAAAT TAATCTAACA	1140
GCTCCAGCAG GAGGAGATCC AGAAGTCACT TTTATGTGGA CAAATTGTCC AGGAGAATTC	1200
TTATATTGCA AAATGAATTG GTTTCTTAAT TGGGTAGAGG ACAGAGACCA AAAGGGTGGC	1260
AGATGGAAAC AACAAAATAG GAAAGAGCAA CAGAAGAAA ATTATGTGCC ATGTCATATT	1320
AGACAAATAA TCAACACGTG GCACAAAGTA GGCAAAAATG TATATTTGCC TCCTAGGGAA	1380
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- 31 -

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## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2571 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

- 32 -

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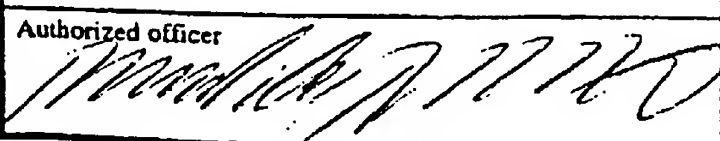
- 33 -

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Applicant's or agent's file reference number	28335/32634	International application No.
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM


(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>6</u> . line s <u>5-9</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution <u>American Type Culture Collection</u>	
Address of depositary institution (including postal code and country) <u>12301 Parklawn Drive</u> <u>Rockville, Maryland 20852</u> <u>United States of America</u>	
Date of deposit <u>1 June 1994</u>	Accession Number <u>69637</u>
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<p>"In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 23(4) EPC)."</p>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
<u>EP</u>	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
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<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer 	Authorized officer

Applicant's or agent's file reference number	28335/32634	International application N
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>7</u> , line s <u>20-23</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit 1 June 1994	Accession Number CRL 11639
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<p>"In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 23(4) EPC)."</p>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
EP	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
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<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
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### CLAIMS

What is claimed is:

1. A recombinant adeno-associated virus genome comprising adeno-associated virus inverted terminal repeats flanking DNA sequences encoding an immunodeficiency virus protein operably linked to promoter and polyadenylation sequences.
2. The recombinant adeno-associated virus genome of claim 1 comprising the cytomegalovirus (CMV) immediate early promoter, the rabbit  $\beta$ -globin intron, the human immunodeficiency virus rev/envelope sequences, and the rabbit  $\beta$ -globin polyadenylation signal.
3. A recombinant adeno-associated virus genome comprising adeno-associated virus inverted terminal repeats flanking DNA sequences encoding a polypeptide selected from the group consisting of tyrosine hydroxylase, aromatic amino acid decarboxylase, nerve growth factor, brain derived neurotrophic factor, NT-3, NT-4/5, glial derived neurotrophic factor and fibroblast growth factor, wherein said DNA sequences are operably linked to promoter and polyadenylation sequences.
4. A DNA vector comprising the recombinant adeno-associated virus genome of claim 1 or 2.
5. The DNA vector according to claim 4 which is vector pAAV/CMV/SIVrev-gp160/neo/rep-cap (ATCC 69637).

6. A DNA vector comprising the recombinant adeno-associated virus genome of claim 3.

7. A mammalian host cell stably transfected with a recombinant adeno-associated virus genome and with adeno-associated virus rep-cap genes.

8. The mammalian host cell of claim 7 wherein said recombinant adeno-associated virus genome is a recombinant adeno-associated virus genome according to claim 1 or 2.

9. The mammalian host cell of claim 8 which is HeLa cell line A64 (ATCC CRL 11639).

10. A mammalian host cell stably transfected with a recombinant adeno-associated virus genome and with adeno-associated virus rep-cap genes, wherein said recombinant adeno-associated virus genome is a recombinant adeno-associated virus genome according to claim 3.

11. A method for producing infectious recombinant adeno-associated virus comprising the step of infecting a host cell according to claim 7 with a helper virus of adeno-associated virus.

12. A method for producing infectious recombinant adeno-associated virus comprising the step of infecting a host cell according to claim 8 with a helper virus of adeno-associated virus.

13. A method for producing infectious recombinant adeno-associated virus comprising the step of infecting a host cell according to claim 10 with a helper virus of adeno-associated virus.

14. Infectious recombinant adeno-associated virus produced by the method of claim 11.

15. Infectious recombinant adeno-associated virus produced by the method of claim 12.

16. Infectious recombinant adeno-associated virus produced by the method of claim 13.

17. A vaccine composition comprising the infectious recombinant adeno-associated virus of claim 14.

18. A vaccine composition comprising the infectious recombinant adeno-associated virus of claim 15.

19. A method for immunizing a host against human immunodeficiency virus comprising the step of administering an immunity-inducing dose of a vaccine composition according to claim 17 to said host.

20. A method for immunizing a host against human immunodeficiency virus comprising the step of administering an immunity-inducing dose of a vaccine composition according to claim 18 to said host.



21. A method for treating a neurodegenerative disorder comprising the step of administering a therapeutically effective dose of an infectious recombinant adeno-associated virus according to claim 16 to a host exhibiting said neurodegenerative disorder.

22. The method of claim 21 wherein said neurodegenerative disorder is selected from the group consisting of Alzheimer's disease, Parkinson's disease, and Huntington's disease.

23. The method of claim 11 wherein said helper virus contains adeno-associated virus rep-cap genes inserted in its genome.

24. The method of claim 12 wherein said helper virus contains adeno-associated virus rep-cap genes inserted in its genome.

25. The method of claim 13 wherein said helper virus contains adeno-associated virus rep-cap genes inserted in its genome.

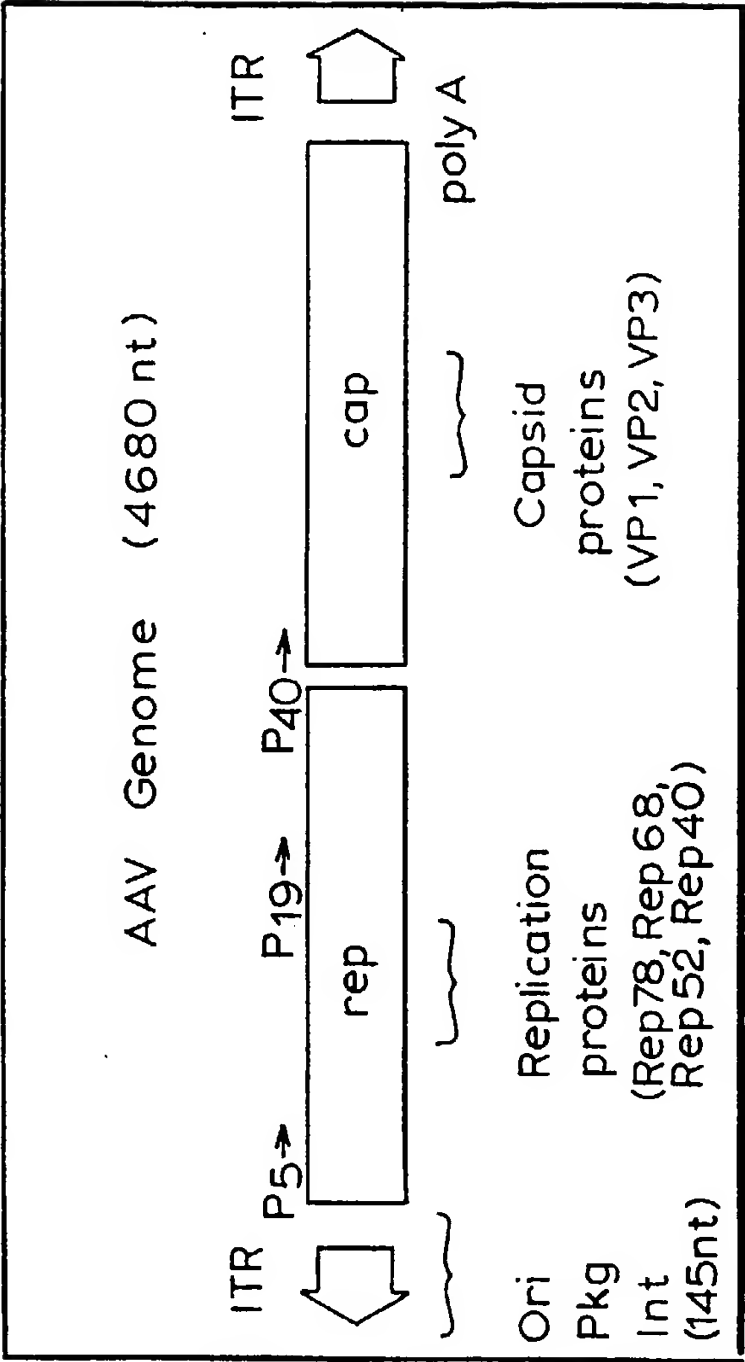


FIGURE 1

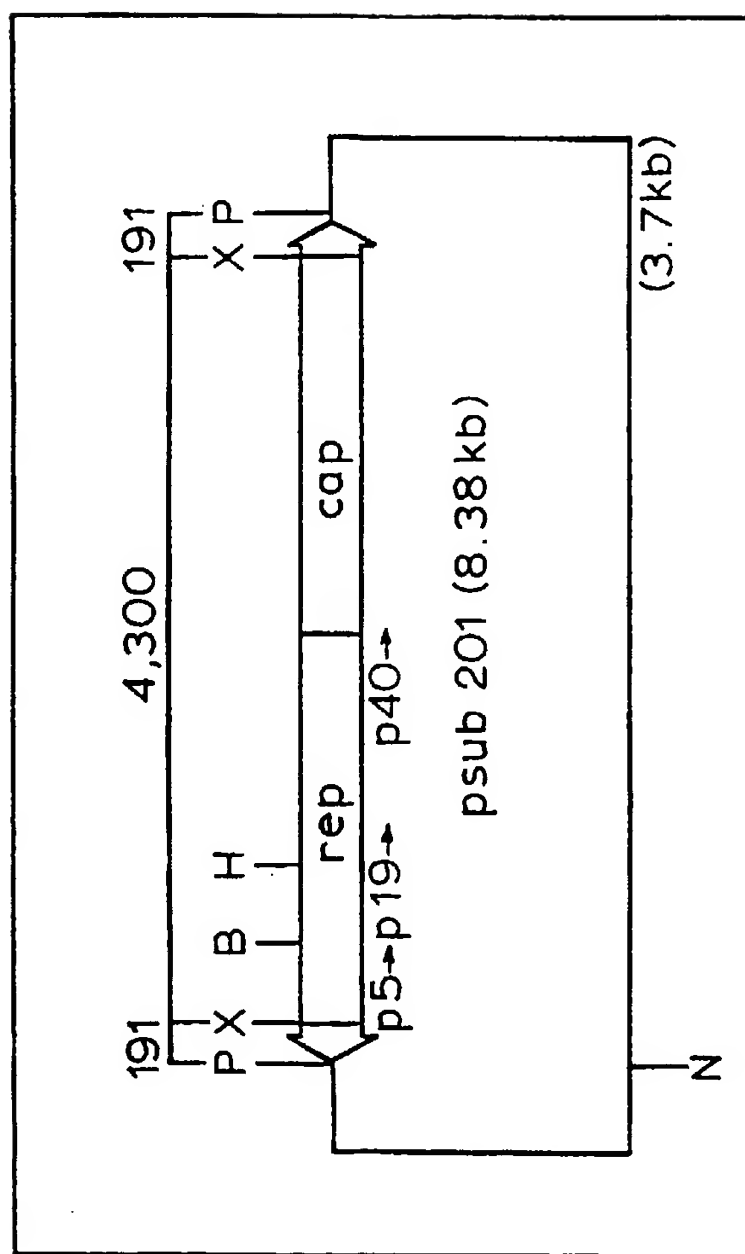
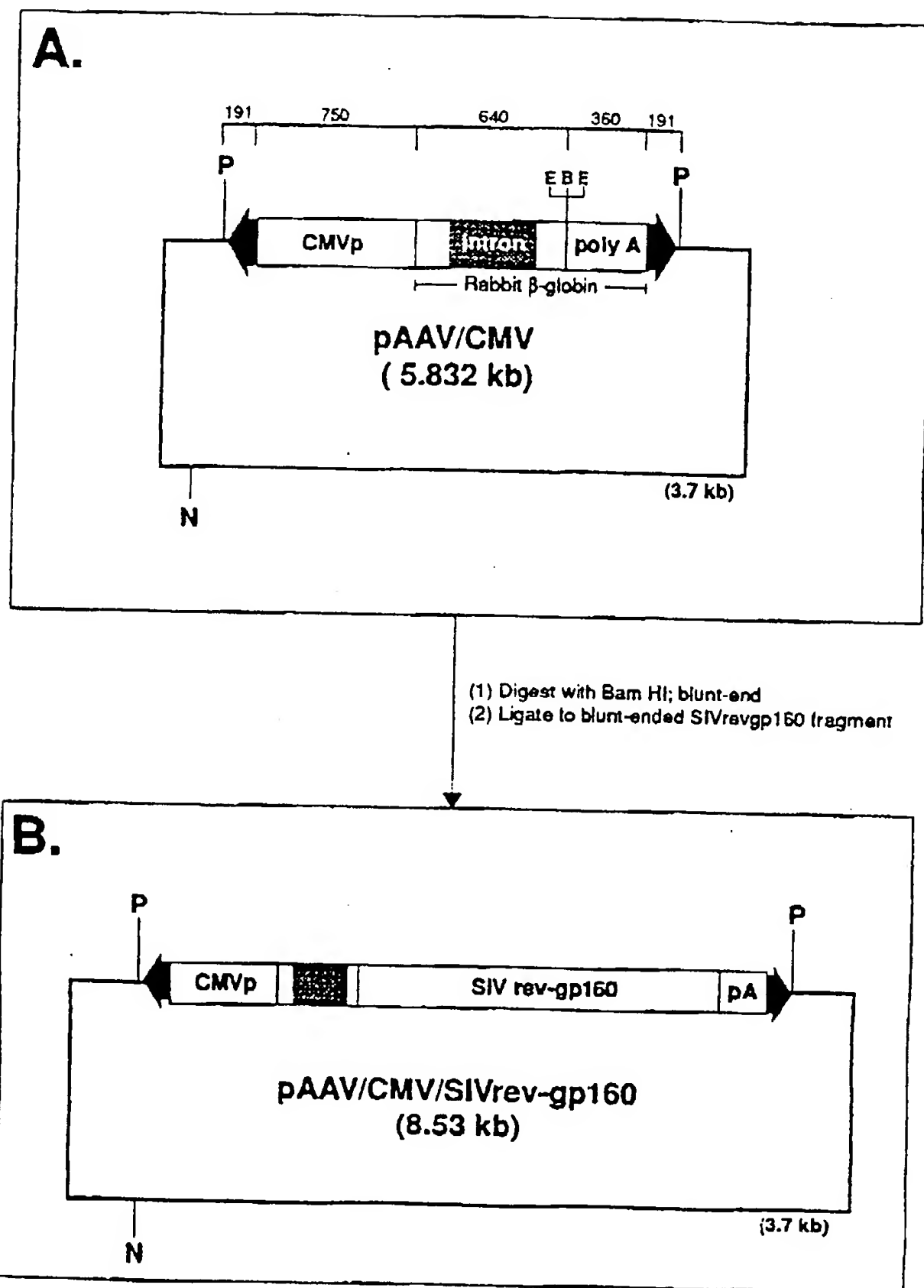
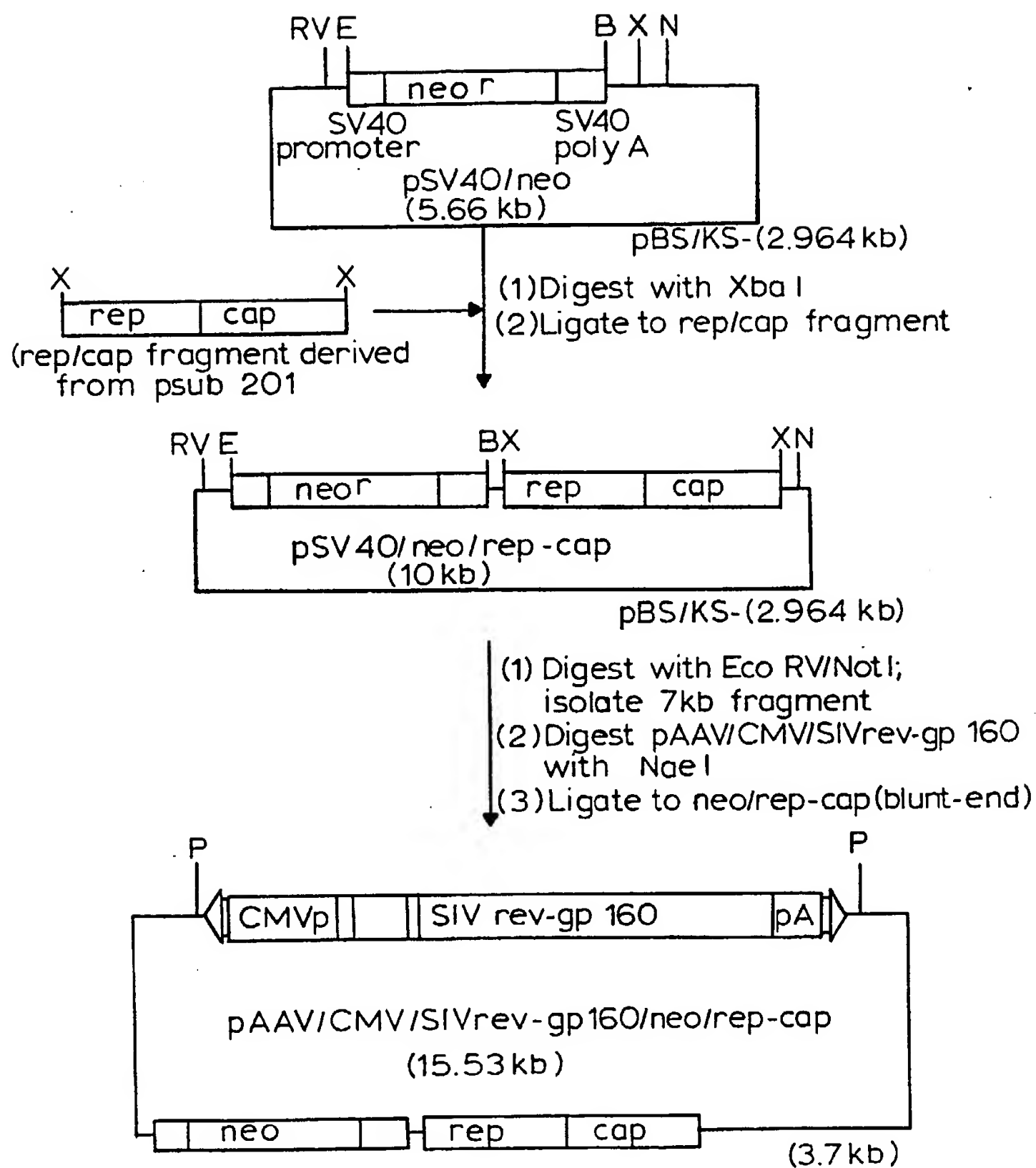


FIGURE 2

FIGURE 3

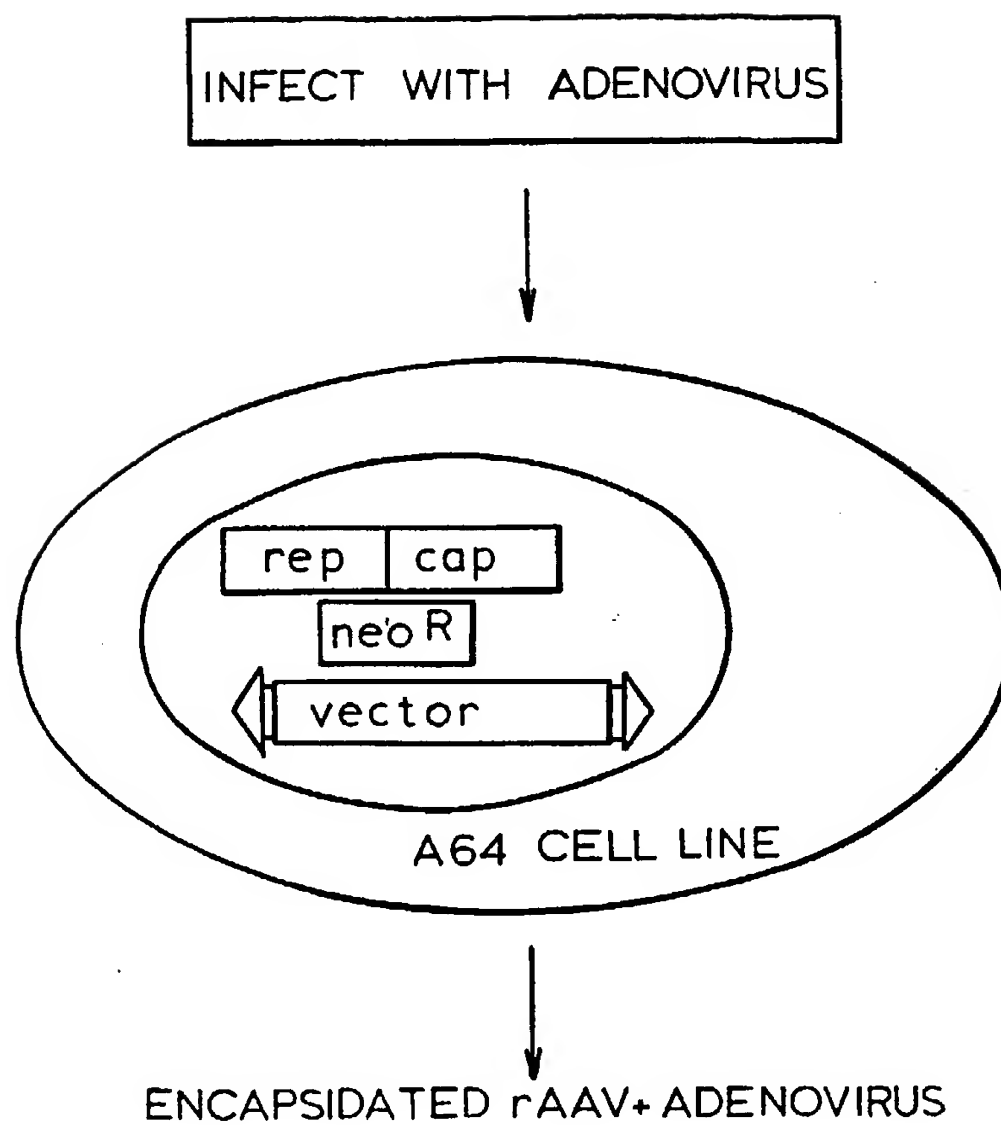


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**FIGURE 4**

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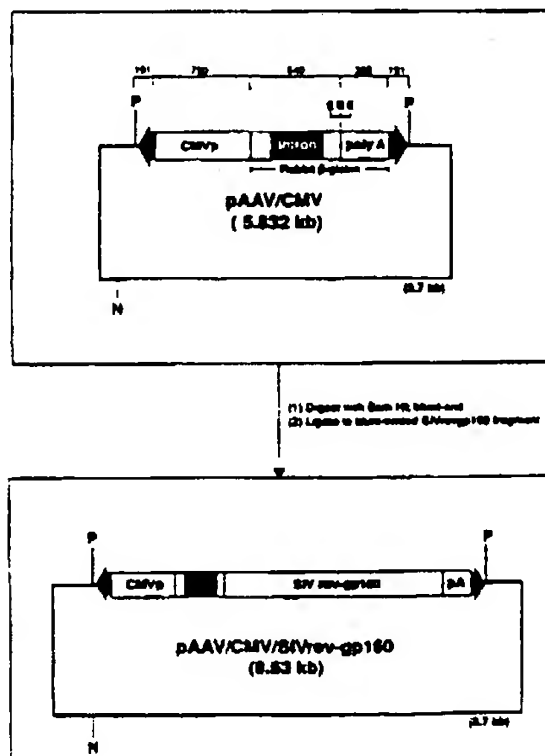
**FIGURE 5**



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/86, C07K 14/16, C12N 15/49, 7/01, 5/10, 15/35, C07K 14/015, A61K 39/21, 39/23, 48/00, C07K 14/155</b>		<b>A3</b>	(11) International Publication Number: <b>WO 95/34670</b> (43) International Publication Date: 21 December 1995 (21.12.95)
(21) International Application Number: <b>PCT/US95/07178</b> (22) International Filing Date: <b>6 June 1995 (06.06.95)</b> (30) Priority Data: <b>08/254,358</b> <b>6 June 1994 (06.06.94)</b> <b>US</b> (71) Applicant: <b>CHILDREN'S HOSPITAL, INC. [US/US]; 700 Children's Drive, Columbus, OH 43205 (US).</b> (72) Inventor: <b>JOHNSON, Philip, R.; 1351 Bread Street, Gahanna, OH 43230 (US).</b> (74) Agent: <b>NOLAND, Greta, E.; Marshall, O'Toole, Gerstein, Murray &amp; Borun, 6300 Sears Tower, 233 South Wacker Drive, Chicago, IL 60606 (US).</b>		(81) Designated States: <b>AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</b>  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: <b>13 June 1996 (13.06.96)</b>	

(54) Title: RECOMBINANT AAV GENOME ENCODING IMMUNODEFICIENCY VIRUS PROTEIN



## (57) Abstract

The present invention provides adeno-associated virus (AAV) materials and methods which are useful for DNA delivery to cells. More particularly, the invention provides recombinant AAV (rAAV) genomes, comprising adeno-associated virus inverted terminal repeats flanking DNA sequences encoding an immunodeficiency virus protein operably linked to promoter and polyadenylation sequences, methods for packaging rAAV genomes, stable host cell lines producing rAAV and methods for delivering genes of interest to cells utilizing the rAAV. Particularly disclosed are rAAV useful in generating immunity to human immunodeficiency virus-1 and in therapeutic gene delivery for treatment of neurological disorders.

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GA	Gabon				



## INTERNATIONAL SEARCH REPORT

onal Application No

PCT/US 95/07178

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 C07K14/16 C12N15/49 C12N7/01 C12N5/10  
C12N15/35 C07K14/015 A61K39/21 A61K39/23 A61K48/00  
C07K14/155

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VACCINES 90: MODERN APPROACHES TO NEW VACCINES, , USA pages 353 - 359 VINCENT, K.A. ET AL. 'Replication and packaging of HIV envelope genes in a novel adeno-associated virus vector system' see the whole document ---	1,2,4,8, 12,15, 18,21
X	WO,A,91 18088 (THE UNITED STATES OF AMERICA) 28 November 1991 cited in the application see page 2, line 35 - page 4, line 16 see page 6, line 15 - line 35 see page 17, line 5 - page 19, line 28 see page 22, line 6 - line 32 see page 23, line 23; claims 1,2,4-7 --- -/--	1,2

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*&\* document member of the same patent family

Date of the actual completion of the international search

26 January 1996

Date of mailing of the international search report

26. 04. 96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
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Authorized officer

CHAMBONNET, F

# INTERNATIONAL SEARCH REPORT

onal Application No

PCT/US 95/07178

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO,A,95 20671 (RHONE-POULENC RORER) 3 August 1995 see page 11, line 6 - line 25 ---	1,2
A	JOURNAL OF BIOLOGICAL CHEMISTRY (MICROFILMS), vol.268, no.5, , MD US pages 3781 - 3790 FLOTTE, T.R. ET AL. 'Expression of the Cystic Fibrosis Transmembrane Conductance Regulator from a novel Adeno-associated virus promoter' see page 3782, column 1, paragraph 2 -----	1

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 95/07178

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 20  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although this claim is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- 9 inventions - see continuation-sheet PCT/ISA/210

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1,2,4,5 and, partially, 8,9,12,15,18,20,24

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US95/07178

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

1. claims 1,2,4,5 and, partially, 8,9,12,15,18,20,24:  
A recombinant adeno-associated virus genome comprising adeno-associated virus inverted terminal repeats flanking DNA sequences encoding an immunodeficiency virus protein operably linked to promoter and polyadenylation sequences, DNA encoding it, mammalian host cells transfected therewith, method of producing it and pharmaceutical composition.
2. partially claims 3,6,10,13,16,21,22,25:  
Subject 1 but limited to sequence encoding Tyrosine hydroxylase
3. idem Subject 1 but limited to sequence encoding aromatic amino acid decarboxylase
4. idem Subject 1 but limited to sequence encoding nerve growth factor
5. idem Subject 1 but limited to sequence encoding brain derived neurotrophic factor
6. idem Subject 1 but limited to sequence encoding NT-3 or NT-4/5
7. idem Subject 1 but limited to sequence encoding glial derived neurotrophic factor
8. idem Subject 1 but limited to sequence encoding fibroblast growth factor
9. claims 7,11,14,17,19,23 and, partially, 8-10,12,13,15,16,20-22,24,25  
A mammalian host cell stably transfected with a recombinant adeno-associated virus genome and with adeno-associated virus rep-cap genes, method of producing infectious recombinant AAV, the infectious viruses and vaccine composition comprising them.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 95/07178

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9118088	28-11-91	AU-B- 7906691 US-A- 5474935	10-12-91 12-12-95
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WO-A-9520671	03-08-95	FR-A- 2716682 AU-B- 1539595	01-09-95 15-08-95
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